

Quick Tests for Tree Seed Viability

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Quick Tests for Tree Seed Viability

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PREFACE

Tree seed quality is usually assessed by means of a standard germination test requiring a minimum of three weeks; with stratification, the test can take six weeks or longer. In some instances standard tests cannot be performed. For example, a nurseryman wanting to sow seeds from last fall's cone crop the following spring finds that seed processing is delayed, so there is not enough time to complete a standard test. Or, a tree breeder needing to check the viability of seeds derived from different male and female parents may have neither the time nor facilities to germinate all of the progeny. In such cases "quick tests" of seed viability can provide reasonably good estimates of seed quality.

The purpose of this manual is to document the procedures of four quick tests commonly used for tree seeds -- the hydrogen peroxide, tetrazolium, X-ray, and X-ray contrast tests. The excised embryo and cutting tests which are also used for evaluating tree seeds, were not considered suitable for this handbook. The excised embryo test depends upon the development of chlorophyll in the embryo, and thus is not a growth test per se. It is effective for broadleaved species which turn green quite readily, but in conifers embryo extension occurs before changes in coloration. The test also relies heavily upon the skill of the analyst, since embryos must be rejected if they become damaged. The cutting test (a longitudinal section exposing the embryo and megagametophyte tissue) was omitted because its primary function is assessing seed development and the presence of seed contents. As a viability test it has proven particularly unreliable.

The manual is written for a wide audience -- researchers, seed technologists, tree breeders, nurserymen, and anyone interested in the rapid assessment of tree seed quality -- and assumes the user has had no previous experience with quick tests. The handbook is divided into four sections, one for each test. Each section contains an introduction explaining the basis for the test and a discussion of other relevant material. Test procedures are augmented with notes and illustrations to assist in test performance and interpretation of results. At the end of each section are suggestions for additional reading.

No single quick test is the best in all situations, and each has its own advantages and disadvantages. Of the four tests, the hydrogen peroxide method

is the only one that actually measures growth. Its primary advantages are objectivity and simplicity, and since it requires fewer man-hours and less equipment than most other viability tests, it is also the least expensive. It does, however, require a full week to complete and can be difficult to perform on small seeds.

The tetrazolium test focuses directly upon the physical and physiological condition of the embryo and endosperm. Since results can be obtained within 8 to 24 hours, tetrazolium is faster than all quick tests except the X-ray method. Its principal drawback is the difficulty in standardization, since it relies heavily upon the expertise and intuition of the experienced analyst. Further, the procedures can be tedious and results may overestimate actual germination.

The X-ray method is the most rapid of the quick tests discussed in this manual. Because only a few minutes are required to produce an X-ray image, a large number of seeds may be examined in a short time. The X-ray test is based upon physical examination of the seeds, but when they are treated with contrast agents, some physiological assessments can also be made. Unfortunately the initial cost of equipment and supplies is high, and seed quality is determined without regard to dormancy.

One should be aware that the term "quick" only refers to the duration of the test, and that quick tests actually require more analyst hours than standard germination tests and are not as dependable. Quick tests usually estimate viability based on traits other than emergence and growth, and because they are performed without consideration for environmental factors, they may only approximate actual germination in the laboratory or in the nursery.

Finally, any assessment of seed quality is only as good as the care with which samples are withdrawn. Improper sampling will render quick test results -- or any other test results -- invalid. Therefore, it is strongly recommended that analysts refer to and be familiar with accepted seed sampling procedures outlined in the International Seed Testing Association Rules for Seed Testing (1976) or Association of Official Seed Analysts Rules for Testing Seeds (1978). These publications can be found in most seed testing laboratories or can be obtained from the above associations.

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I would especially like to acknowledge the assistance of the staff at the Oregon State University Seed Testing Laboratory, particularly Betty Bond and Rodger Danielson, who have had extensive experience with testing tree seeds and have very generously contributed to the procedures section.



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HYDROGEN PEROXIDE METHOD

Introduction

Hydrogen peroxide (H_2O_2) was first used to rapidly assess viability in malting barley (Parker and Hill 1955), but it was soon adopted as a quick test for tree seeds. In their study of eight Pacific Northwest conifers, Ching and Parker (1958) found that H_2O_2 results and germination tests correlated significantly, yet H_2O_2 tests took only 7 days as compared to 60 days for standard tests. Aware of the rapidity with which results could be obtained, the authors suggested that the H_2O_2 test be considered whenever time was a limiting factor.

The primary advantages of the H_2O_2 method are that it is objective and technically simple. It is performed by clipping the seed coat and incubating the seeds in a dilute H_2O_2 solution for one week, at which time seeds with radicles longer than 1 mm are counted and the test is terminated. Because it is the only method that actually measures growth, the H_2O_2 test is often chosen in preference to the tetrazolium, X-ray, and excised embryo methods (Danielson 1972). The test is the least expensive to perform because it requires fewer man-hours than other viability tests.

The biochemical basis for the H_2O_2 test is still uncertain, but it is believed that H_2O_2 enhances the early phases of germination by stimulating respiration. Hydrogen peroxide is an unstable compound which decomposes readily into oxygen and water at room temperature. Although the reaction can be retarded by reducing light and temperature, it is probably the oxygen end product that is responsible for various properties of H_2O_2 .

In seeds, H_2O_2 may induce germination by acting as an electron acceptor in respiratory electron transport, or by oxidizing endogenous inhibitors. However, the most likely mechanism is that the spontaneous decomposition of H_2O_2 increases ambient oxygen levels, resulting in the oxidation of storage products and the initiation of germination. This view has been supported by the experimental evidence that H_2O_2 stimulates the β -oxidation of fatty acids in Douglas-fir seeds (Ching 1959). In tree

seeds, the mobilization of fatty acid reserves is one of the earliest events of germination.

Elevated oxygen levels from H_2O_2 decomposition may also account for the effectiveness of H_2O_2 as a sterilant, in which capacity it frequently has been used against infection by micro-organisms (Trappe 1961; Neal et al. 1967; Riffle and Springfield 1968; Barnett 1976). The observation that H_2O_2 can substitute for the stratification requirement of many tree seeds is probably also related to the effects of high oxygen concentrations on seed metabolism (Ching and Parker 1958; Carter and Jones 1962; Kozlowski and Torrie 1964; Hedderwick 1969; Lebrun 1970; Henrotte and Devillez 1976).

The primary disadvantage of the H_2O_2 test, as with the tetrazolium test, is that viability is rated independently of environmental factors, and therefore may not reliably predict seed performance in the field. Seed dormancy and stratification requirements may also be difficult to assess because the test can be used with comparable results on both dormant and non-dormant seedlots. Another problem is its impracticality for small seeds. The test can be effectively performed on large seeds such as ponderosa and Jeffrey pine, Douglas-fir, and the true firs, but for small seeds such as western hemlock and western redcedar, the tetrazolium test is generally preferred (B. Bond, Oregon State Seed Laboratory, personal communication, 1980).

These objections notwithstanding, the advantages of the H_2O_2 test probably outweigh its disadvantages. Test results may sometimes be slightly lower than those of a standard germination test, but since the test requires only inexpensive materials and minimal expertise, it is a good choice for analysts with few resources and limited experience in seed evaluation.

General Procedure

1. Soaking

Withdraw 300 seeds using random sampling techniques, and soak the seeds in 100 mL 1.0% H_2O_2 at room temperature (20°C) overnight (Figure 2). Only four replications of 50 seeds each are needed for the test, but extra seeds should be included to replace ones damaged during cutting.

2. Cutting

Using a single edge razor blade, cut the tip off the radicle end of the seed deep enough to expose, but not damage, the radicle tip. The cut should be made at an angle -- not straight across -- in order to reduce damage to the radicle (Figure 1). Place the seeds in water after cutting to prevent dessication.

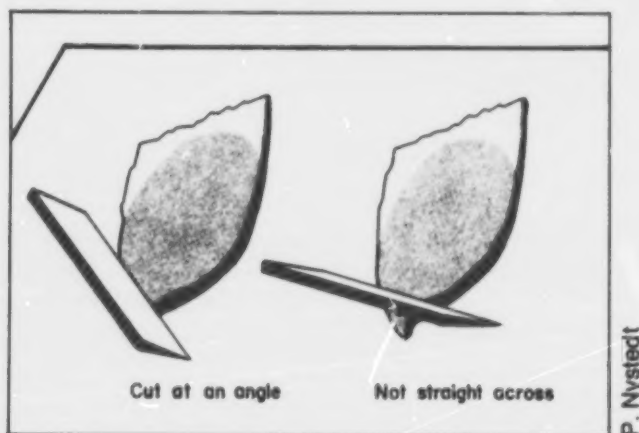
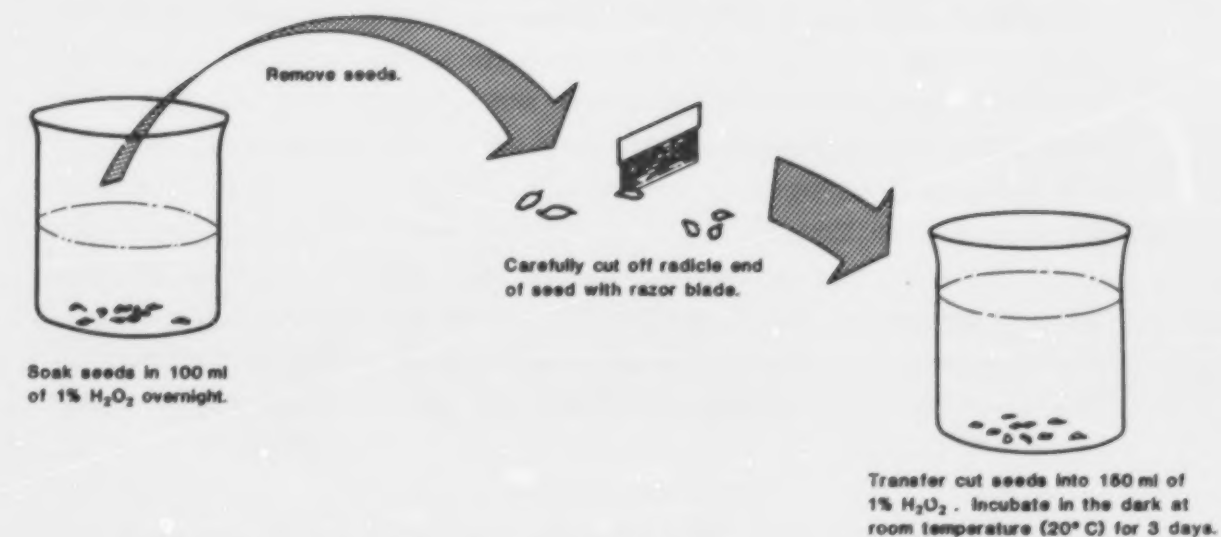


FIGURE 1. Cutting diagram (hydrogen peroxide test).

Small seeds such as spruce, lodgepole pine, and western hemlock are difficult to handle. Special care is needed during cutting (especially western hemlock) to ensure that the radicle is not damaged when the seed tip is removed.

For true fir species, note that pitch released from seeds may cause them to stick to one another or to the sides of the container.



HYDROGEN PEROXIDE QUICK TEST

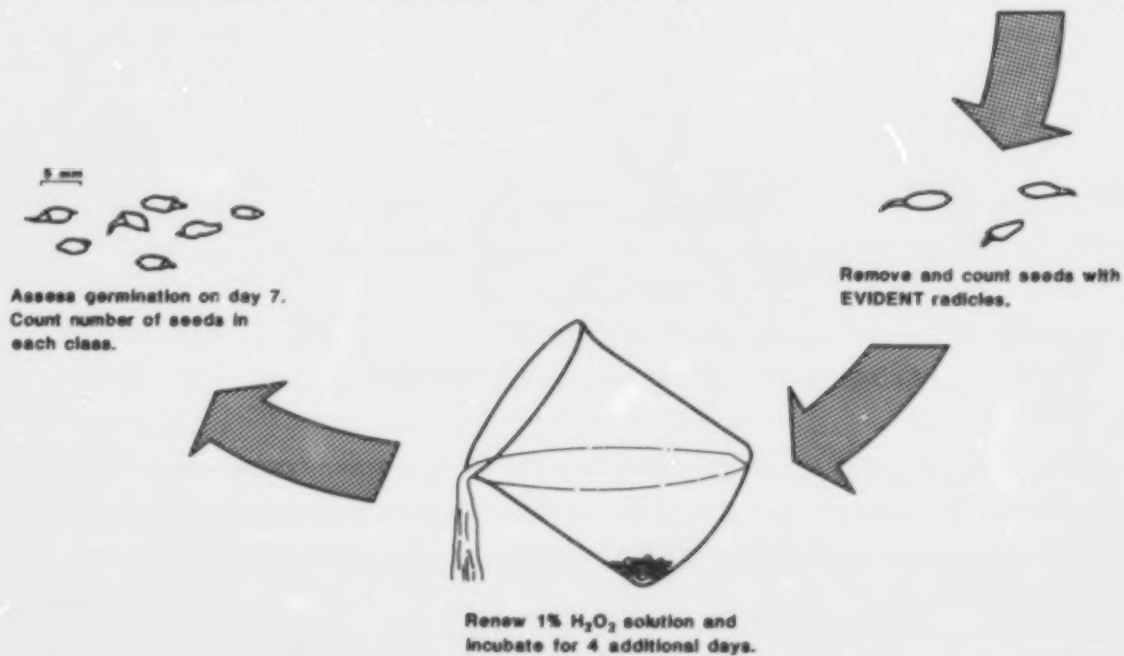


FIGURE 2. Hydrogen peroxide test procedure.

3. Incubation

Pour 150 mL 1.0% H_2O_2 (for large seeds, 200 mL) into each of four beakers. Add 50 cut seeds per beaker and incubate in the dark at 20-25 °C to allow embryo elongation to occur.

4. Assessment

After 3 days incubation, remove and count any seeds with "evident" radicles (Table 1). Return remaining seeds to a fresh H_2O_2 solution. On day 7 remove and assess all seeds (Table 1, Figure 3), and terminate the test. Data is generally expressed as the proportion of "evident" germinants to total seeds used in the test.

TABLE 1. Assessment classes (hydrogen peroxide test)

<u>Class</u>	<u>True fir spp</u>	<u>Other species</u>
Evident	Radicle > 5 mm long	Radicle > 1 mm long
Slight	Radicle < 5 mm long	Radicle < 1 mm long
None	No radicle protrusion	No radicle protrusion

Notes:

- Some radicles may curl during elongation. The total length of the radicle should be taken into account for purposes of classification.
- Embryos may separate from the seed coats during soaking, especially if too much of the coat was removed during cutting. If this occurs, every effort should be made to match the free embryos with empty seeds in order to avoid errors in classification. For assessment purposes, very long embryos should be classified as "evident", but small embryos must be removed from the tally. If a substantial number of embryos cannot be classified, repeat the test.

Douglas-fir

(Pseudotsuga
menziesii)
8x



Evident



Slight

Amabilis fir

(Abies amabilis)
6x



Evident



Slight

FIGURE 3. Seed assessment (hydrogen peroxide test)

Comparison of H_2O_2 Results and Germination

To determine the reliability of the H_2O_2 test, seven seed sources each of amabilis fir, white spruce, lodgepole pine, Douglas-fir, and western hemlock were germinated and simultaneously tested with H_2O_2 . Accuracy of the H_2O_2 test was checked by comparing percent germination under standard conditions with percent "evidents" (Figure 4).

Hydrogen peroxide estimated viability of three species reasonably well, but greatly underestimated seed potential of the other two species. For amabilis fir, white spruce, and lodgepole pine, differences between H_2O_2 results and actual germination averaged about 4.0%, but for Douglas-fir, H_2O_2 results were 19.8% less than controls, and were 39.0% less for western hemlock.

The results demonstrate that the H_2O_2 test, as with any viability test, must be used with caution until responses of individual species have been ascertained. The failure to predict performance for Douglas-fir and western hemlock does not exclude the use of the test for these species, but only indicates that H_2O_2 did not perform well under the conditions or with the seed sources chosen for the comparison. With further research and adjustments in test condition or techniques, the predictive ability of the test may be improved.

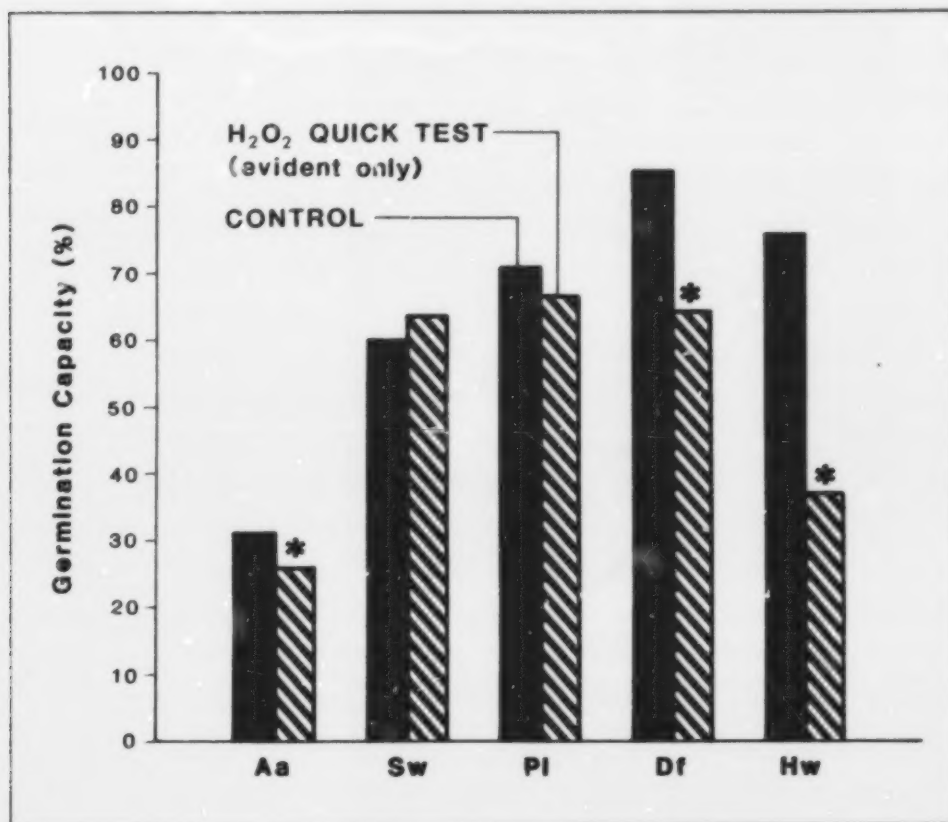


FIGURE 4. Comparison of actual and predicted germination (hydrogen peroxide).

Controls were stratified for 3-4 weeks at 2°C, then germinated under 8-hr days at 30°C/20°C (or 20°C constant for western hemlock) for 3-4 weeks in an incubator. Treatment means are based on seven seedlots with four replications per treatment. Percentages marked with an asterisk are predictions that were significantly different from controls at $p = 0.05$ using Duncan's Multiple Range test. Species abbreviations are Sw=white spruce (*Picea glauca*); Df=Douglas-fir (*Pseudotsuga menziesii*); Pl=lodgepole pine (*Pinus contorta*); Hw=hemlock (*Tsuga heterophylla*); Aa=amabilis fir (*Abies amabilis*).

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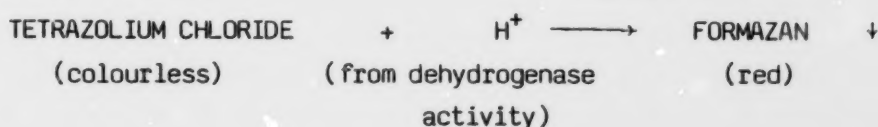
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TETRAZOLIUM METHOD

Introduction

The tetrazolium (TZ) technique defines live and dead areas of the embryo and endosperm by differential, topographic staining. The chemical basis for the tetrazolium method is the reaction:



When seeds are incubated for 4-16 hours in tetrazolium chloride solution, dehydrogenase enzymes in actively respiring areas of the embryo and endosperm produce hydrogen ions which react with the colourless TZ to form the red-coloured formazan, which precipitates in and stains the tissues. By evaluating the location and intensity of the formazan stain, one can readily differentiate between healthy and damaged portions of the seed.

The tetrazolium test was developed through the efforts of many international workers (Moore 1969). In 1922, Turina in Yugoslavia discovered that living cells in germinating seeds could reduce the colourless salts of selenium and tellurium to the red or black colour of the pure elements. At the same time in Russia, Neljubon, working independently on vital staining using indigo carmine, was the earliest author to appreciate that staining patterns in the endosperm and embryo might be used to evaluate the normality and vigour of essential seed structures. Although his work was not recognized for many years, Hasegawa of Japan published results in 1931 which indicated that he was aware that vital staining with selenium and tellurium salts could be used to make inferences about seed health and vigour; thus he probably deserves the credit for being the first to develop vital staining into a practical, rapid test for tree seeds.

The usefulness of vital stains was greatly improved in 1940 by the development of the topographical method, a technique that combines the use of

various stains with the evaluation of how the stains are distributed within the seed. Lakon (Germany) demonstrated that the location and degree of staining could be correlated with seed vigour. Although he originally used selenium salts for his topographical method, selenium was too poisonous for practical work. His search for a similar but non-poisonous compound led him to change to 2, 3, 5-triphenyltetrazolium chloride, the form of tetrazolium still used for seed testing.

Information on the tetrazolium test was not disseminated during the war years until Allied forces visited Lakon's laboratory in 1945, but thereafter adoption of the tetrazolium method was still slow because the test was not well understood. In North America the major proponent of the method has been Moore, the author of many papers on seed quality testing, who has stressed that the TZ test possesses a number of advantages not found in other tests. He claims that with careful analysis of tetrazolium staining patterns, an experienced worker can find weaknesses not observed in the standard germination test. Unlike the germination test, the TZ evaluation is not confounded by other factors; it focuses directly upon the physical and physiological condition of the embryo and endosperm, completely bypassing seed-environment interactions. Other than an X-ray, TZ is faster than other quick tests, for within 8 to 24 hours both vigour and viability information can be obtained from a single TZ test. The stained seeds can also be preserved for later re-evaluation.

The principal drawback of the technique is that the test is hard to standardize, since it relies heavily upon the expertise and intuition of the experienced analyst. Further, because the TZ rating is independent of inhibitions imposed by dormancy, results are not necessarily indicative of actual performance. With tree seeds this is an important consideration, since many require some pretreatment to break dormancy. The TZ test often rates more seeds as viable than what actually germinate in the standard incubator test (Kaul and Zentsch 1969; Simak 1970). Moore believes that the TZ test accurately assesses seed germination potential, whereas the standard germination test only demonstrates how the seed performs under incubator conditions. However, incubator tests have proven to be more relevant to the

nurseryman because they usually correlate directly or can be corrected to predict field performance. A final reason for limited acceptance is that the procedures can be very tedious since cutting and evaluating each seed can be very time-consuming.

The utility of the test depends upon whether the analyst is more interested in ultimate potential, or in assessing how seeds will perform under field conditions. In spite of its disadvantages, the tetrazolium method is frequently chosen because inherent seed traits can be measured directly and results can be obtained very quickly.

General Procedure

1. Soaking

Using random sampling techniques, obtain four representative samples of 50 seeds each. Include a few extra seeds to replace those that might be damaged during preparation. Soak seeds in water overnight at room temperature (20°C) to soften them for cutting and to hydrate the tissues.

Drain excess water but place seeds on wet paper towels to keep them moist throughout cutting and assessment.

2. Cutting

The cutting technique used will depend upon the size and shape of the seeds, or the method that is most comfortable for the analyst. For large seeds such as the true firs, cuts should be relatively large to hasten staining, but care must be taken not to damage the embryo.

Method A. Hold the seed between thumb and forefinger, and starting at one end and orienting the blade edge perpendicular to the long axis (Figure 5A.1), slice a thin layer off the endosperm (Figure 5A.2). The advantage to this method is that imbibed seeds are less likely to be crushed during cutting.

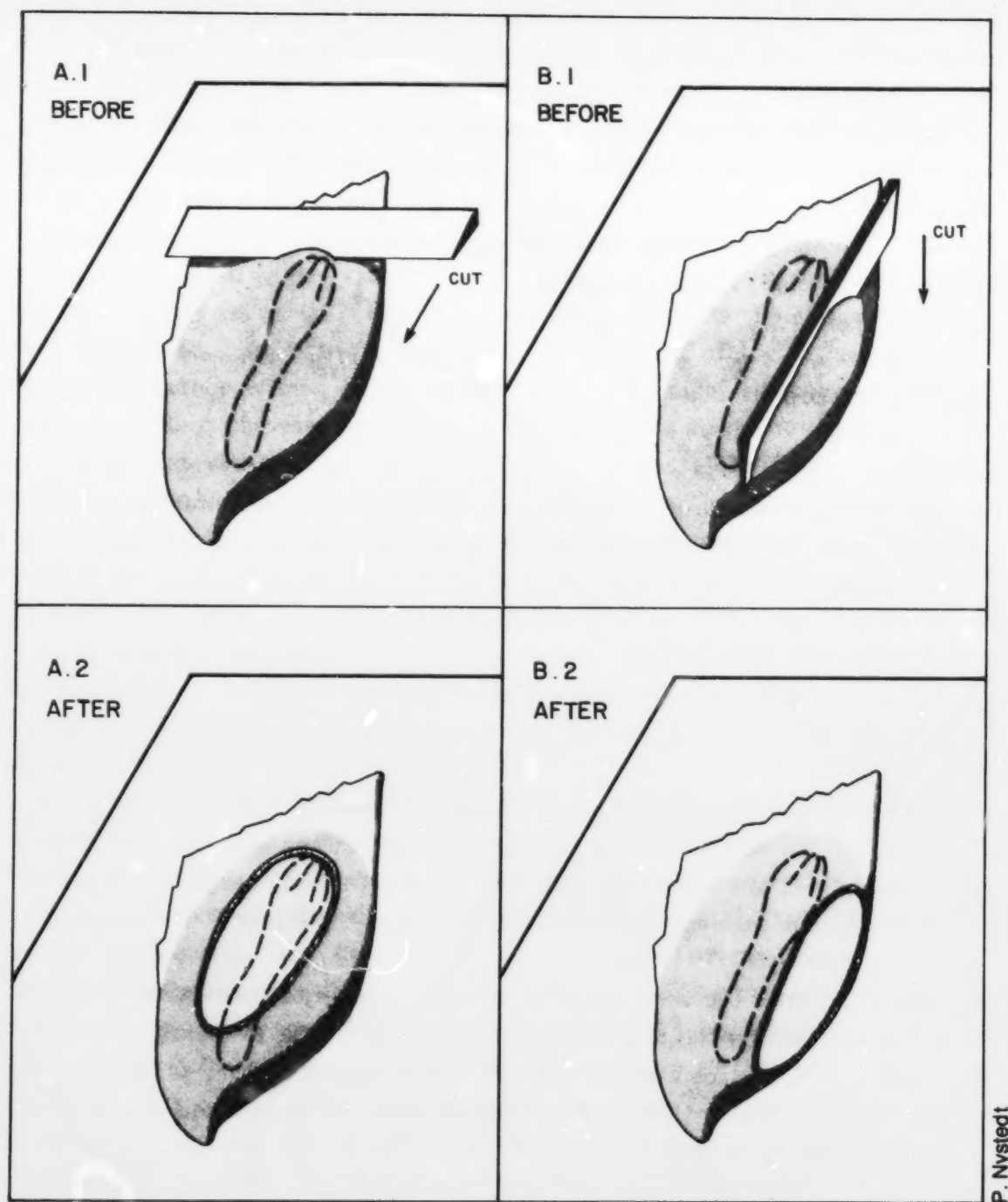


FIGURE 5. Cutting diagram (tetrazolium test).

Method B. Place seed on a flat surface, then make a cut parallel to the long axis of the seed, but slightly off centre to avoid damaging the embryo (Figure 5B.1 and 2). This method is probably better for small seeds such as western hemlock, which are more difficult to hold.

Method C. Cut a small portion off both ends of the seed, taking care not to injure the embryo. This method is reported to result in better penetration of stain in Douglas-fir and true fir seeds (G. Eicke, Bayrische Forstliche versuchs- und Forschungsanstalt, München, personal communication, 1982).

3. Incubation

Place cut seeds into suitable containers, e.g., watch glasses, small petri dishes, or vials. Provide enough 1% TZ solution¹ (pH 6.5-7.0) to cover seeds and to allow for evaporation.

Incubate seeds at 38°C in the dark for 2-8 hours. The time required for adequate staining varies with the species and seedlot; extra seeds should be included to monitor the progress of the staining. Too dark a stain should be avoided since this may mask weakened tissues. Seeds are easier to evaluate when they are light pink to red.

When staining is complete, immediately drain the TZ solution and rinse seeds with water two to three times to remove any unbound formazan. Cut seeds in half to excise the embryos and expose the remaining endosperm. After embryos are separated from endosperms they should be turned over so that the uncut side can be seen, as the sliced surface often does not stain well.

4. Evaluation

Evaluate the seeds using the criteria in Table 2 and referring to Figures 6 through 11. It is important that seeds be kept moist throughout the evaluation.

¹ Prepared from 2,3,5-triphenyl tetrazolium chloride in tap water or buffer solution. Stored at 5°C in the dark.

TABLE 2. Topographic stain evaluation (tetrazolium test)

<u>Class</u>	<u>Description</u>	<u>Viability</u>	<u>Figure</u>
1	Embryo completely stained	Germinable	6
2	Very pale staining	Possibly germinable	-
3	Cotyledons unstained	Non-germinable or possibly germinable	7 10
4	Radicle unstained	Non-germinable or probably not germinable	9 11
5	No staining	Non-germinable	8

Essential growth centres called meristems must be well stained for a seedling to grow. In the embryonic root, the meristem is located near the radicle tip, while in the shoot, growth derives from the base of the cotyledons. An embryo may survive if there is weak staining in these areas, but will probably succumb if exposed to stress.

Before tabulating seed viability, it is helpful to place all well-stained embryos in a group separate from poorly stained embryos. Referring to these two groups will aid in the classification of "questionable" embryos.

Damaged seeds normally do not stain well, but when tested soon after damage, injured tissues may stain more intensely than the surrounding healthy areas (Moore 1964, Moore 1966). If seeds stain unevenly look for other signs of damage or deterioration, such as tissue which appears flaccid, lacks luster, or shrinks rapidly upon exposure to air. However, the lack of uniformity may simply be due to poor penetration of stain, in which case cutting and incubation procedures may have to be adjusted.

Experience is the major criterion for success in evaluating seeds with the TZ method. A practiced analyst can make predictions based on general anatomical features and obvious staining differences, but considerable experience with tetrazolium chloride is needed to be able to assess the quality of seeds that have been weakened but not killed by aging, heat, freezing, or microbial infection.

Viability Assessment:

A. Viable

1. Embryo

- completely stained embryo fills at least 75% of the cavity.
- necrotic areas on non-meristematic tissue comprise < 25% of surface area and do not pass through the diameter of the embryo.
- tissue is firm with a smooth surface.
- occasionally radicle and cotyledon tips will not stain; however the seed may still be classed as viable, but only if staining progresses gradually towards tips without any sharp demarcations or signs of necrosis, and the endosperm meets viability requirements.

2. Endosperm

- tissue is firm with a smooth, even texture.
- necrotic and damaged areas < 25% of surface area are evenly stained and not in contact with the embryo. (Unlike angiosperm seeds, the "endosperm" of gymnosperms will stain.) Care must be used in evaluating unstained areas since intensity and progress of staining varies with the time and effectiveness of the initial cut.

B. Non-viable (non-germinable; includes recognizable abnormalities)

1. Embryo

- vital areas such as apical meristem and radicle tip are not stained.

FIGURES 6-11. Tetrazolium staining.

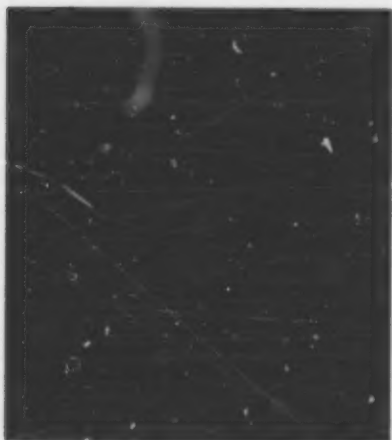


FIGURE 6. Normal, fully stained embryo. Uniform red colour indicates all tissues are healthy and respiring. --Germinable (Class 1). (Pinus contorta)

FIGURE 7. Radicle is well stained, but main axis and cotyledons are completely without stain, therefore incapable of shoot growth. --Non-germinable (Class 3). (Tsuga heterophylla)



FIGURE 8. Stain absent. No evidence of any respiratory activity. --Non-germinable (Class 5). (Tsuga heterophylla)



FIGURE 9. Weak staining in radicle in area of the meristem. --Probably not germinable (Class 4). (Pseudotsuga menziesii)



FIGURE 10. Cotyledons well stained, but 40% of embryo only weakly stained. --Probably not germinable (Class 4). (Picea glauca)



FIGURE 11. Poor stain in shoot. Meristem may not be affected. --Possibly germinable (Class 3). (Pinus contorta)

- embryo is not firm with a smooth even texture.
- necrotic or dark-stained areas comprise > 25% of surface area or pass through the diameter of embryo.
- embryo is damaged, e.g., with cracks and bruises.
- there are one or several embryos, none of which are > 50% of cavity.

2. Endosperm

- tissue appears soft or mushy.
- necrotic and damaged areas comprise > 25% of surface area.
- there is uneven or spotty staining.

C. Empty

- seeds lack endosperm and/or embryo.
- includes insect-, wood-, and pitch-filled seeds.

Comparison of TZ Results and Germination

To determine how well the tetrazolium test correlated with germination, seven seedlots each of amabilis fir, white spruce, lodgepole pine, Douglas-fir, and western hemlock were evaluated by the two methods (Figure 12).

On average, TZ results were 9.8% greater than germination tests for amabilis fir and white spruce, and 5.0% greater for lodgepole pine. These results agree with those of other investigators. Since TZ stains viable seeds without regard to dormancy status, it has been found to overestimate germination, especially in seeds that require pretreatment to germinate (Grano 1958; Kaul and Zensch 1969; Simak 1970; Mason et al. 1982).

In both of the other two species chosen for comparison, TZ underestimated germination. Tetrazolium results were 14.2% less (significantly different at $p = 0.05$) for Douglas-fir, and 7.8% less for western hemlock. The magnitude of these differences may render the TZ test unsuitable for applications where a degree of precision is necessary, however, relative to the hydrogen peroxide and X-ray contrast methods, TZ results corresponded more closely with germination percentages, and thus may be the best alternative for quickly estimating viability when standard tests cannot be performed.

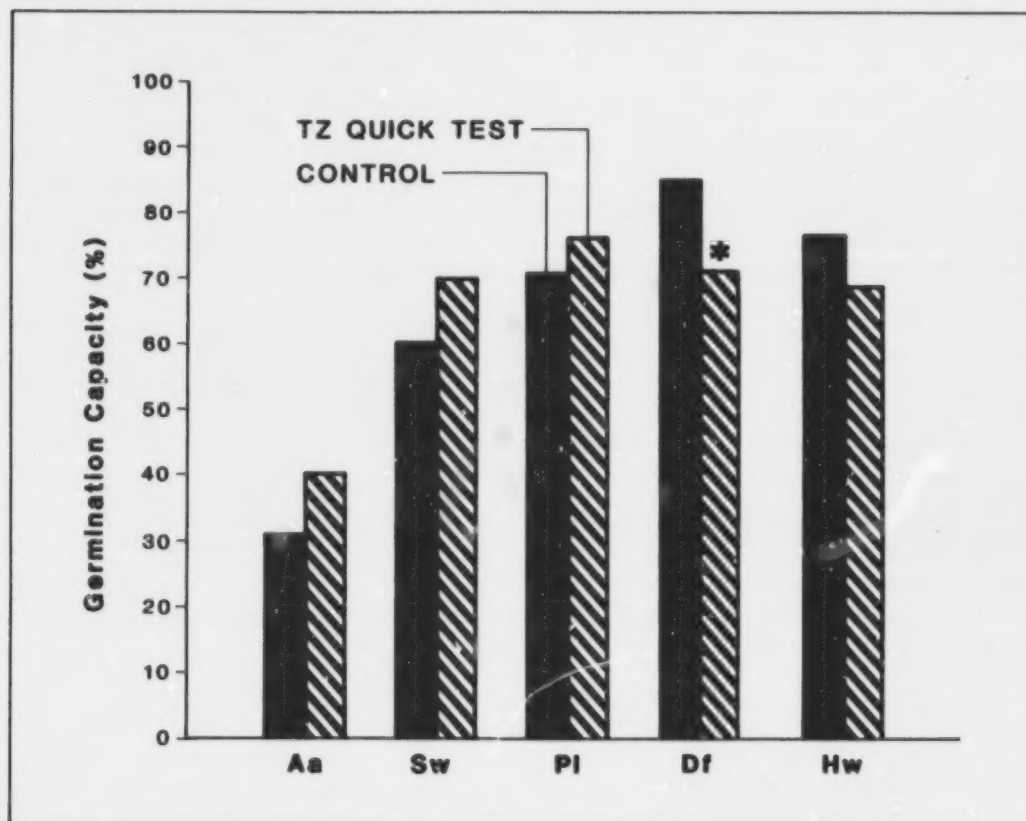


FIGURE 12. Comparison of actual and predicted germination (tetrazolium test).

Controls were stratified for 3-4 weeks at 2°C, then germinated under 8-hr days at 30°C/20°C (or 20°C constant for western hemlock) for 3-4 weeks in an incubator. Treatment means are based on seven seedlots with four replications per treatment. Percentages marked with an asterisk are predictions that were significantly different from controls at $p = 0.05$ using Duncan's Multiple Range test. Species abbreviations are Sw=white spruce (*Picea glauca*); Df=Douglas-fir (*Pseudotsuga menziesii*); Pl=lodgepole pine (*Pinus contorta*); Hw=western hemlock (*Tsuga heterophylla*); Aa= amabilis fir (*Abies amabilis*).

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X-RAY METHOD

Introduction

The first use of X-rays for seed quality testing was not an overwhelming success. Professor A.N. Lundstrom (Uppsala University), addressed a meeting of the Silvicultural Society of North Sweden in 1903 and stated that:

In order to decide whether cones from certain districts ought to be collected for seeding, the speaker had used roentgen rays. Some seed then appeared dark, others light. The latter ones showed themselves to be incompletely filled and to contain more or less air; they were dull. This method could not be used in practice, however, since it would be too tedious and troublesome.

Considering these pessimistic remarks, it is not surprising that X-rays did not quickly gain acceptance among most seed workers. However, a number of entomologists soon recognized the effectiveness of radiography for detecting insects, and adopted the technique for screening a variety of plant materials for infestation (Yuasa 1926; Fenton and Waite 1932; Shevchenko 1937; Milner et al. 1950, 1952; Nicholson et al. 1953).

Although still widely used for insect studies, X-rays are now primarily used for tracing seed development, and for evaluating the effects of genetic and environmental factors on developmental processes. This shift in the application of X-rays was largely due to the work of Müller-Olsen et al. (1956). To facilitate seed X-ray analysis, they devised a classification system which rated the condition of the embryo and the endosperm and correlated the X-ray ratings with germination results. Gustafsson and Simak (1956) believed that X-ray interpretation should be standardized internationally, and promoted their classification scheme to support this concept.

For the rapid determination of seed quality, X-rays have several advantages over traditional methods of seed vigour testing. In a review of the topic, Heydecker (1969) commented: "A [seed vigour] test should be sound in principle, easy and cheap to perform on a large number of samples in rapid

succession, and obviously yield reproducible results which can be objectively assessed, clearly recorded, and meaningfully interpreted." The X-ray method fulfills most of these criteria. It is perhaps the most rapid of the quick tests discussed in this manual. Since it takes only a few minutes to produce an X-ray image, a large sample of seeds may be examined in a short time. The test is based upon the physical examination of the embryo and endosperm, and when they are treated with contrast agents (discussed in the following section), physiological assessments can also be made. It takes relatively little training to produce an X-ray, but some experience is needed to interpret the images.

Although the initial outlay for an X-ray machine, film, and processing equipment is relatively high, thereafter the cost of materials compares favourably with most standard methods of quality testing. The X-ray method has been successfully used for a number of years for testing seeds of many different species (Table 3). It has been demonstrated to be a reliable seed viability indicator, showing good correlations with results obtained in standard germination tests (Leadem 1981). Interpretation and reliability of the X-ray method have been improved by the introduction of standard criteria for embryo and endosperm development and for staining by contrast agents (Müller-Olsen *et al.* 1956; Kamra 1971). Because radiographs are permanent records, X-rays can also be re-evaluated at a later date if necessary.

There are some disadvantages to the X-ray method, the most notable being the difficulty with determining quality in relation to seed dormancy. However, this shortcoming also applies to the tetrazolium chloride and hydrogen peroxide methods. Although X-rays used in seed testing laboratories are "soft" (low energy) radiation and are therefore safer than the "hard" X-rays used in medical applications (Kamra and Simak 1965), all personnel operating X-ray equipment should be instructed in its safe use and must comply with all safety regulations (see section on Safety).

TABLE 3. Species in which x-radiography has been used to evaluate seed quality

<u>Common name</u>	<u>Scientific name</u>	<u>References</u>
e. white pine	<u>Pinus strobus</u>	Belcher (1974)
autumn olive	<u>Elaeagnus umbellata</u>	
yellow poplar	<u>Liriodendron tulipifer</u>	
bald cypress	<u>Taxodium distichum</u>	
Norfolk Is. pine	<u>Araucaria heterophylla</u>	
Cornus spp.	<u>Cornus</u>	
Sonderregger pine	<u>Pinus sonderreggeri</u>	
longleaf pine	<u>P. palustris</u>	
loblolly pine	<u>P. taeda</u>	
-	<u>Acaena adscendens</u>	Dorne, Devillez, and
-	<u>Azorella selago</u>	Courtemanche (1974)
-	<u>Philica nitida</u>	
-	<u>Ranunculus bitermatus</u>	
-	<u>Uncinia compacta</u>	
Douglas-fir	<u>Pseudotsuga menziesii</u>	Edwards (1973)
w. hemlock	<u>Tsuga heterophylla</u>	
yellow cypress	<u>Chamaecyparis nootkatensis</u>	
limber pine	<u>Pinus flexilis</u>	
cotton	<u>Gossypium hirsutum</u>	Fenton and Waite (1932)
sugar beet	<u>Beta vulgaris</u>	Grimm (1958)
Scots pine	<u>Pinus silvestris*</u>	Gustafsson and Simak (1956)
Norway spruce	<u>Picea abies*</u>	
redwood	<u>Sequoia sempervirens</u>	Hansen and Muellder (1963)
sugar beet	<u>Beta vulgaris</u>	Hogaboam (1961)
cucumber	<u>Cucumis sativus</u>	Kamra (1964 a)
melon	<u>Cucumis</u> spp.	--- (1967)
teak	<u>Tectona grandis</u>	--- (1973)
60 spp. of tropical forestry seed		--- (1974)
squash	<u>Cucurbita pepo</u>	--- and Bhargava (1967)
pumpkin	<u>Cucurbita</u> spp.	
celery	<u>Apium graveolens</u>	--- and Bhargava (1967)
white spruce	<u>Picea glauca</u>	Klaehn and Wheeler (1961)
wheat	<u>Triticum aestivum</u>	Milner et al. (1950, 1952a)
rice	<u>Oryza sativa</u>	--- (1952 b)
safflower	<u>Cathamus tinctorius</u>	Morgan and Maguire (1974)
eggplant	<u>Solanum Melongena</u> , var. <u>esculentum</u>	Nakamura (1974)
pepper	<u>Capsicum</u> spp.	
33 spp. including peas, beans, cotton, bird-cherry, rose		Shevchenko (1937)
soya bean	<u>Glycine max</u>	Singh and Banerjee (1968)
16 spp. of agricultural and horticultural seeds		Swaminathan and Kamra (1961)

* Plus 54 other forest spp.

General Procedure

1. Sample Preparation

Obtain four representative samples of 100 seeds from each lot or treatment to be examined. Wings and debris should be removed from the samples to eliminate undesirable details in the X-ray.

Seeds can be placed directly on the X-ray film or photographic paper, but for most routine work a holder is generally used. Holders may be clear plastic boxes or compartmented trays. Sometimes trays with small cavities are used when seeds must be studied individually, but any material intended for use as a seed holder should first be tested for X-ray penetrability. A simple holder can be constructed with clear acrylic plastic (e.g., Plexiglas) for the frame, and acetate film (e.g., clear document covers) for the base. It is convenient to divide the holder into compartments to separate individual samples. Seeds may also be fixed to sticky tape to keep them separated.

Radiographic paper is normally used when a permanent record is not required. Paper is less expensive than X-ray film and can be processed quickly in instant processors, but it gives relatively poor image resolution. Although images can be made permanent by processing X-ray paper in photographic fixers (hypo baths), celluloid based X-ray films have sharper resolution and provide a better permanent record. The disadvantage is that X-ray films take longer to process. If funds are available, there are machines, used primarily in medical applications, that will give a dry film within minutes, but these units are relatively expensive. In either case, you should choose the material that is most suitable for your purposes and circumstances. Also remember that both film and paper are light sensitive and must be handled in darkness or under a photographic safelight before processing.

2. Factors Affecting the Quality of the X-Ray Image

Kilovoltage (kV) is the measure of voltage potential within the X-ray tube. An increase in kV will produce shorter wavelength ("hard") X-rays.

The kV affects the contrast of the image; a lower kV improves the contrast, while a higher kV reduces the density differences.

Milliamperage (ma) is the measure of the current applied to the tube. Increasing the ma increases the number of X-rays produced in a given time. The ma influences the density, but not the contrast of the image. A high ma will overexpose (darken) the image.

Exposure time is the time during which the specimen is exposed to the X-rays for making the radiograph. Exposures should be expressed as milliamperere-seconds (mas) or milliamperere-minutes (mam). By changing the exposure time the density of the image will be altered. In choosing an exposure time it must be remembered that there is an interaction between exposure time and ma. To retain the same image quality any increase in exposure time requires a proportional decrease in ma. For example, an exposure of 100 mas, obtained with a tube current of 5 ma and an exposure time of 20 seconds, produces the same image density as an exposure made at 10 ma for 10 seconds.

The distance between the focal spot (or target) and the film surface is the Focus-Film-Distance (FFD) (sometimes referred to as the target-film distance, TFD). An increase in the FFD decreases the intensity of the radiation according to the inverse square law. Changes in FFD influence the sharpness of the image.

The distance between the object and the film surface (OFD) affects the image quality. The greater the distance, the poorer the image, as details will be less distinct. If fine detail is necessary the seeds may be placed directly on the film surface, although in routine work the film is usually kept in a film carrier to make handling easier.

Note: Potential X-ray users should study the Kodak Manual, "Radiography in Modern Industry" (1980), which explains these factors more thoroughly.

3. X-Ray Exposure and Development

Place the seeds (with or without a holder) on top of the film or paper in the X-ray machine.

Make the exposure. Individual X-ray machines will probably require different exposure times and voltage settings to produce the best image. Settings will also vary for different species. For the best results, a time-voltage-exposure series should be made whenever a different machine or material is being used.

Develop the film or paper. Paper is usually developed in instant processors which produce a print within a few seconds. Film must be developed in a darkroom.

4. Evaluation

Evaluate the seeds according to the seed development criteria given in Table 4. Note any insects or damage. Seeds are classified as follows:

A. Viable seeds

- well-filled seeds with distinct embryos.
- the length of the embryo in relation to the cavity should be noted (Classes 1 and 3). (Figure 14)

B. Poor quality seeds

- of questionable viability; they have indistinct or distorted embryos, very small embryos, and may exhibit shrinkage of the endosperm and embryo (Classes 2, 4, 5, and 6). (Figure 18)

C. Non-viable seeds

- seed coat with no contents, shrivelled contents, or missing embryos (Classes 7, 8 and 9). (Figures 15, 16, 17)

D. Broken, cracked or shattered seeds

- breaks may be in the seed coat, endosperm, embryo, or in several of these areas. (Figures 21, 22)
- should be classed with non-viable seeds.

E. Insect larvae

- may be present in the seeds. (Figures 19, 20)
- should be classed with non-viable seeds.

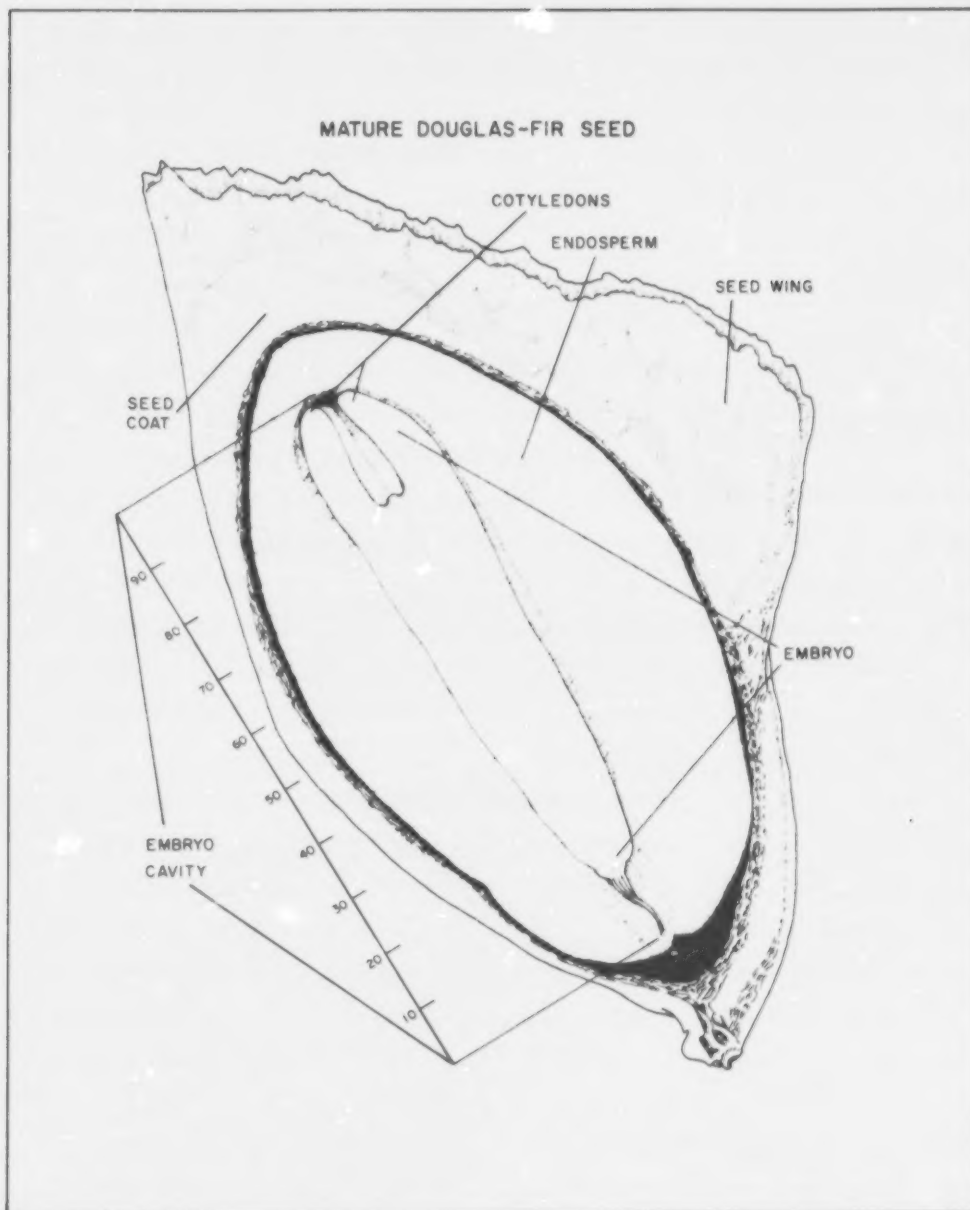


FIGURE 13. Anatomy of a mature Douglas-fir seed.

TABLE 4. Seed development criteria (X-ray test)*

Class	Description		Figure
	Embryo	Endosperm	
1	FULLY DEVELOPED. Fills or almost fills embryo cavity. Germinable.	FULLY DEVELOPED. Fills or almost fills seed coat; absorbs X-rays well.	14
2	FULLY DEVELOPED. Fills or almost fills embryo cavity. Questionably viable.	SHRUNKEN OR DEFORMED. Not filling seed coat completely.	-
3	50% to 75% OF CAVITY. Not wholly developed. Germinable.	FULLY DEVELOPED. Fills or almost fills seed coat; absorbs X-rays well.	-
4	50% to 75% OF CAVITY. Not wholly developed. Questionably viable.	SHRUNKEN OR DEFORMED. Not filling seed coat completely.	18
5	< 50% OF CAVITY. One or several embryos, none longer than half of the cavity. Questionably viable.	FULLY DEVELOPED. Fills or almost fills seed coat; absorbs X-rays well.	-
6	< 50% of CAVITY. One or several embryos, none longer than half of the cavity. Questionably viable.	SHRUNKEN OR DEFORMED. Not filling seed coat completely.	-
7	NO EMBRYO. Non-germinable.	FULLY DEVELOPED. Fills or almost fills seed coat; absorbs X-rays well.	-
8	NO EMBRYO. Non-germinable.	SHRUNKEN OR DEFORMED. Not filling seed coat completely.	16, 17
9	NO EMBRYO. Empty seed. Non-germinable.	NO ENDOSPERM. Empty seed.	15, 16
10	INSECT-FILLED OR DAMAGED SEEDS. Embryo $\pm$ Non-germinable.	Endosperm $\pm$.	19, 20 21, 22

* modified after Müller-Olsen et al. (1956)

5. Safety

Since this technique involves the use of X-radiation, safety should be a prime concern of the operator. The following recommendations should be observed at all times:

1. The X-ray operator should wear personal radiation monitors whenever using the X-ray machine.
2. The kVp control should be turned to zero immediately after and between each exposure.
3. The door to the X-ray machine should be closed at all times except during brief loading periods.
4. The key should be turned to the OFF position except during warm-up and exposure.
5. The machine should be checked periodically to be sure the X-ray beam shuts off when the door is opened. Use photographic film, pocket dosimeter or other suitable radiation detector. Promptly service if radiation leakage is detected.
6. Have machine regularly monitored by radiation protection personnel.

FIGURES 14-22. X-ray seed assessment. All X-rays were taken along the longitudinal axis of the seeds. Species shown are Abies amabilis (Figures 19, 20) and Pinus contorta (Figures 14-18 and 21-22).

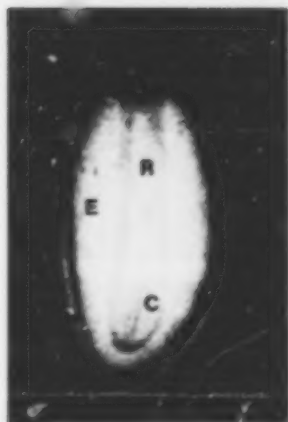


FIGURE 14. Normal, fully developed seed. Embryo completely fills the embryo cavity. Cotyledons (C) and radicle (R) can be easily seen. Gametophytic tissue (endosperm, E) surrounds and provides nourishment for the developing seedling. The seed coat(S) is the hard outer covering that protects the fleshy endosperm and embryo. --Germinable (Class 1).



FIGURE 15. Empty seed. Neither endosperm or embryo has developed, but the seed coat is clearly visible. --Non-germinable (Class 9).



FIGURE 16. Undeveloped seed. There is no embryo development and the endosperm has developed only slightly. --Non-germinable (Class 8?, 9?).

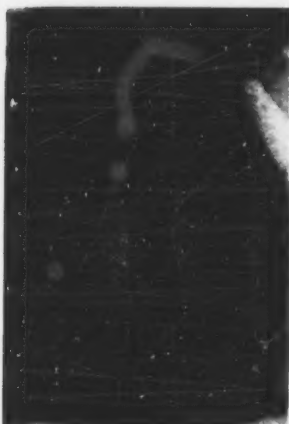


FIGURE 17. Incompletely developed seed. The endosperm has developed, but does not completely fill the seed. The embryo is missing. --Non-germinable (Class 8).



FIGURE 18. Incompletely developed seed. The endosperm does not fill the seed and the embryo occupies only half of the embryo cavity. In some instances, seeds of this type can be allowed to mature so that full development can occur. --May be germinable (Class 4).



FIGURE 19. Insect damage. A seed chalcid larva (Megastigmus spp) has consumed the entire contents of the seed. --Non-germinable (Class 10).



FIGURE 20. Insect damage. An insect has eaten part of the endosperm, but the embryo remains intact. It is possible that this seed might germinate if sown, but it probably would be subject to deterioration in storage from fungus or other pathogens.
--Questionably viable (Class 10).

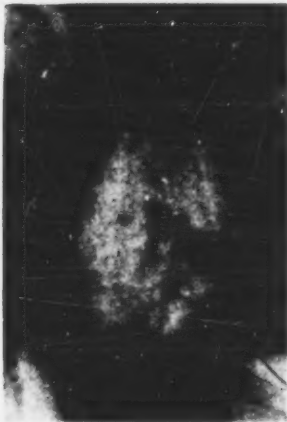


FIGURE 21. Mechanical damage. Cracks can be seen in the endosperm and the embryo, but the seed coat is still entire.
--Non-germinable (Class 10).



FIGURE 22. Mechanical damage. A large portion of the seed coat is missing, and the inner contents of the seed have been broken in half.
--Non-germinable (Class 10).

X-RAY CONTRAST METHOD

Introduction

A further step towards improving the X-ray technique was the addition of contrast agents to enhance the interpretation of radiographs. The biological basis for contrast agents is that they "stain" dead or damaged tissues, but are unable to pass through the semi-permeable membranes of healthy living cells. These agents absorb radiation and their accumulation in damaged tissues causes a stronger shadow, i.e., increased contrast in the radiographic image. According to the location and the area of impregnation within the seed, it is possible to estimate its germinability.

Initially, aqueous solutions of compounds containing heavy cations (e.g., BaCl_2 , AgNO_3) were used to impregnate seeds prior to radiographic examination (Simak 1957). Later, Kamra (1963a) showed that it was possible to estimate the amount of mechanical damage to Scots pine seeds with the help of the X-ray contrast agents, urografin, and umbradil. Although urografin and umbradil had previously been used for human radiography, they proved unsatisfactory for routine X-ray assessment because of their high cost and the fact that in most countries these compounds could only be purchased with a doctor's prescription.

As a result, Kamra (1971) was prompted to seek other staining agents. Using Norway spruce seeds, he tested aqueous solutions of the halides sodium iodide, potassium iodide, sodium bromide, potassium bromide, and lithium bromide. Kamra found that while iodides generally gave better contrast than bromides at equal concentrations, a 15-minute soak in 40% NaI gave the best contrast of all the combinations tested. However, the use of inorganic iodides and bromides is not without problems. Seeds can be killed by prolonged exposure to the solutions. If seeds are not washed, chemicals adhering to the seed coat can cause misleading artifacts, and if seeds are washed for too long, contrast agents impregnating damaged areas might be removed.

Considering the difficulties with halogen and heavy cation compounds, vaporous contrast agents have been suggested to replace aqueous agents (Simak 1974a). Because they absorbed X-radiation well and exhibited rapid mobility through biologic tissues, chlorinated alkanes such as chloroform (trichloromethane) proved to be good vaporous contrast agents. Chloroform is superior to aqueous agents such as BaCl_2 in a number of ways. Impregnation with chloroform appears as single diminutive dots or coherent spots, whereas BaCl_2 impregnation occurs as large spots, or covers the entire seed. Because the edges of chloroform-stained spots are sharper than those of BaCl_2 , chloroform gives greater contrast between impregnated and unimpregnated areas. Staining with vaporous agents is also easier because the seeds are suspended in the vapours rather than being soaked in solution.

After comparison of the two agents, Simak (1974a) concluded that chloroform staining was more sensitive than BaCl_2 , and that chloroform could not only disclose seeds which are germinable, but those which are just beginning to deteriorate. Early detection of deterioration would be especially beneficial to assess seed quality after processing (e.g., after extraction or dewinging) and after prolonged storage. However, vaporous contrast agents cannot necessarily be used with equal success in all species. In parallel studies, Simak (1974a) also found that vaporous alkanes stained Pinus silvestris well, but only mechanically damaged seeds of Picea abies were impregnated. Thus the applicability of the vaporous X-ray contrast method to different species must be examined before screening of seed quality is attempted.

As stated in the previous section on the X-ray method, there are some disadvantages to any radiographic technique, but these can be reduced or eliminated if proper precautions are taken. Specifically with the X-ray contrast method, prolonged exposure to contrast agents will reduce seed viability, and therefore, treatment times should be carefully monitored.

General Procedure

1. Staining

Place seed sample in a cheesecloth bag. Tie with a string and suspend bag over 100 mL of chloroform (trichloromethane) in a closed container or Erlenmeyer flask for 2 hours.

2. X-Radiography

Remove seed samples from vapours. Chloroform evaporates quickly so seeds should be X-rayed within 15 minutes. X-ray an additional seed sample which has not been exposed to chloroform to gauge natural brightness of the embryo and endosperm. In true firs, be careful to note whether the staining is inside the seed or located in the resin pockets.

3. Evaluation (Refer to Table 4).

A. Stained, damaged, and empty seed

A seed is considered stained (non-viable) if staining occurs in > 25% of the projected surface area of the endosperm or embryo, or if staining occurs in a vital area of the embryo such as the apical meristem or radicle tip. Staining usually originates in three areas of the seed:

- a) the micropyle -- a natural opening at the radicle end of the seed
- b) cotyledon end -- point of attachment of seed wing (may indicate damage during dewinging)
- c) crack in seed coat -- usually stains area around opening.

Staining can occur in small spots or as large bright areas, usually with a clear demarcation between bright and dull areas. Look for staining of the membrane surrounding the endosperm, or around any opening such as a crack or the micropyle. Seeds of the true firs and western hemlock contain resin ducts; these may absorb chloroform and interfere with the evaluation of endosperm and embryo quality.

Note any damaged seed, even if it has not been stained. Damage appears as a crack in the seed coat, endosperm, and sometimes the embryo, and is often associated with heavy staining in the immediate area.

Empty seeds lack an endosperm and embryo. This category includes insect-, wood-, and pitch-filled seeds. It can also include insect-damaged seeds if there has been damage to the embryo that does not fit into the stained category.

B. Non-stained seeds

Non-stained seeds are probably germinable and should be evaluated according to the embryo and endosperm classification scheme (Table 4).

Comparison of X-C Results and Germination

Agreement between X-ray contrast and germination data was evaluated by testing seven seedlots each of amabilis fir, white spruce, lodgepole pine, Douglas-fir, and western hemlock (Figure 23).

Correlation was good for the small-seeded species; average differences between quick test and germination test results were 3.8%, 4.3%, and 7.2% for western hemlock, white spruce, and lodgepole pine, respectively. Much poorer agreement was noted for large-seeded species; X-C results differed 18.8% for amabilis fir and 28.6% for Douglas-fir.

The above results confirm the suitability of X-C for small seeds, but argue against its use for large seeds such as Douglas-fir and amabilis fir. It has previously been mentioned that the absorption of contrast agents by resin vesicles of true firs may complicate the interpretation of X-C radiographs, but another problem with large seeds may be lack of permeation, which would require adjustments in the length of time seeds are exposed to the vapours.

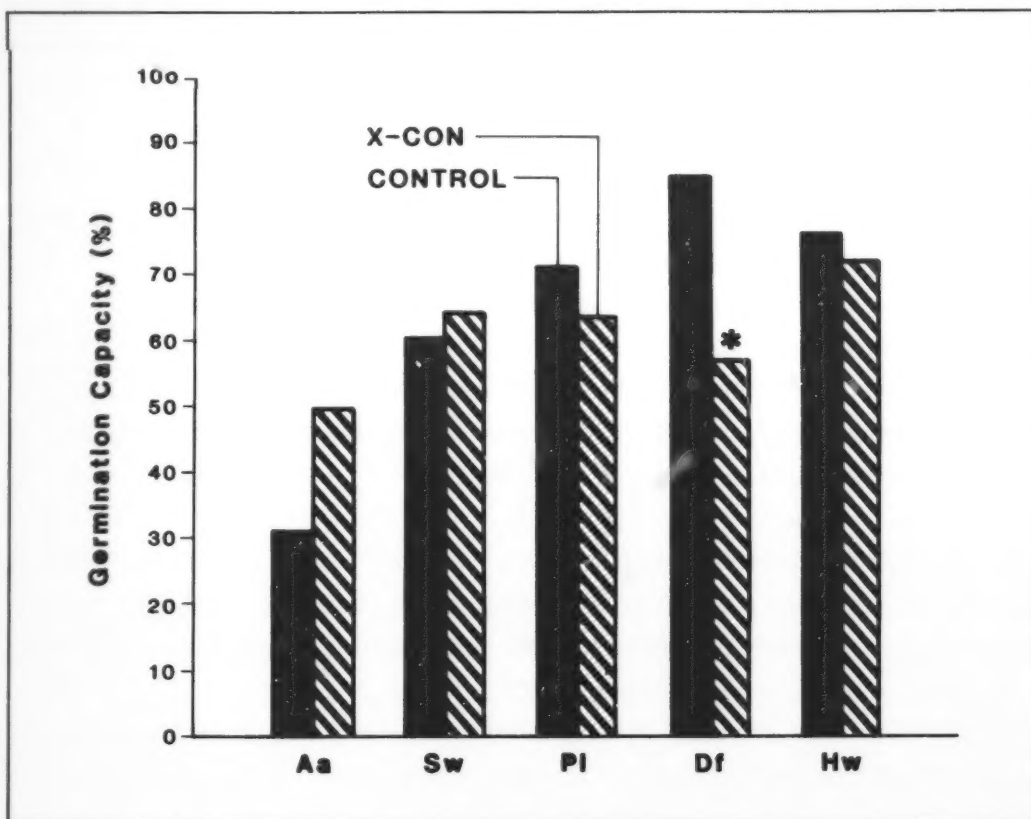


FIGURE 23. Comparison of actual and predicted germination (X-ray contrast)

Controls were stratified for 3-4 weeks at 2°C, then germinated under 8-hr days at 30°C/20°C (or 20°C constant for western hemlock) for 3-4 weeks in an incubator. Treatment means are based on seven seedlots with four replications per treatment. Percentages marked with an asterisk are predictions that were significantly different from controls at $p = 0.05$ using Duncan's Multiple Range test. Species abbreviations are Sw=white spruce (*Picea glauca*); Df=Douglas-fir (*Pseudotsuga menziesii*); Pl=lodgepole pine (*Pinus contorta*); Hw=western hemlock (*Isuga heterophylla*); Aa=amabilis fir (*Abies amabilis*).

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APPENDIX

Scientific Names of Species Referred to in the Text

<u>Common Name</u>	<u>Scientific Name</u>
amabilis fir	<u>Abies amabilis</u> (Dougl.) Forbes
Norway spruce	<u>Picea abies</u> (L.) Karst
white spruce	<u>Picea glauca</u> (Moench) Voss
lodgepole pine	<u>Pinus contorta</u> Dougl. ex Loud.
Douglas-fir	<u>Pseudotsuga menziesii</u> (Mirb.) Franco
western hemlock	<u>Tsuga heterophylla</u> (Raf.) Sarg.